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The effect of the decoy molecule PA401 on CXCL8 levels in bronchoalveolar lavage fluid of patients with cystic fibrosis

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Running title: PA401 decreases CXCL8 in CF airway samples

Abbreviations: CF, cystic fibrosis; BALF, bronchoalveolar lavage fluid; GAGs, glycosaminoglycans; FEV1, forced expiratory volume in 1 second.

Abstract

Background: The chemokine interleukin-8 (CXCL8) is a key mediator of inflammation in airways of patients with cystic fibrosis (CF). Glycosaminoglycans (GAGs) possess the ability to influence the chemokine profile of the CF lung by binding CXCL8 and protecting it from proteolytic degradation. CXCL8 is maintained in an active state by this glycan interaction thus increasing infiltration of immune cells such as neutrophils into the lungs. As the CXCL8-based decoy PA401 displays no chemotactic activity, yet demonstrates glycan binding affinity, the aim of this study was to investigate the anti-inflammatory effect of PA401 on CXCL8 levels, and activity, in CF airway samples *in vitro*.

Methods: Bronchoalveolar lavage fluid (BALF) was collected from patients with CF homozygous for the *ΔF508* mutation (n=13). CXCL8 in CF BALF pre and post exposure to PA401 was quantified by ELISA. Western blot analysis was used to determine PA401 degradation in CF BALF. The *ex vivo* chemotactic activity of purified neutrophils in response to CF airway secretions was evaluated post exposure to PA401 by use of a Boyden chamber-based motility assay.

Results: Exposure of CF BALF to increasing concentrations of PA401 (50-1000 pg/ml) over a time course of 2-12 hours *in vitro,* significantly reduced the level of detectable CXCL8 ($P<0.05$). Interestingly, PA401 engendered release of CXCL8 from GAGs exposing the chemokine susceptible to proteolysis. Subsequently, a loss of PA401 was observed ($P<0.05$) due to proteolytic degradation by elastase like proteases. A 25% decrease in neutrophil chemotactic efficiency towards CF BALF samples incubated with PA401 was also observed ($P<0.05$).

Conclusion: PA401 can disrupt CXCL8:GAG complexes, rendering the chemokine susceptible to proteolytic degradation. Clinical application of a CXCL8 decoy, such as PA401, may serve to decrease the inflammatory burden in the CF lung *in vivo*.

Keywords: Cystic fibrosis, bronchoalveolar lavage fluid, glycosaminoglycan, interleukin-8/CXCL8, antagonist decoy, neutrophils.

1. Introduction

Cystic fibrosis (CF) is the most common lethal genetic disease in Caucasians that is caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR) chloride channel (Riordan et al., 1989). CF patients suffer from persistent pulmonary infections accompanied by chronic neutrophil-dominated inflammation that results in severe progressive lung injury. Mechanisms for how reduced CFTR function leads to chronic lung disease in CF include altered ion transport across the airway epithelium and dehydration of the airway surface layer (Matsui et al., 1998). The dehydrated airways are lined with thick mucus which contains elevated levels of anionic glycosaminoglycans (GAGs) produced on the bronchial epithelial cell surface (Reeves et al., 2011a). GAGs are unbranched, O-linked linear polysaccharide chains normally linked to a core protein (to form proteoglycans) located on the cell surface of almost all eukaryotic cell types (Adage et al., 2012; Rek et al., 2009). The immobilization of chemokines, specifically CXCL8 by GAGs plays a major role in the establishment of haptotactic gradients that contribute to the recruitment of leukocytes through the endothelium during inflammatory exacerbations (Proudfoot et al., 2003). GAGs in the lung are located on bronchial cells in the interstitial space between the capillary endothelium and the alveolar epithelium and the most abundant include heparan sulphate (HS) and chondroitin sulphate (CS) (Solic et al., 2005; Suki et al., 2005). In lung tissue CXCL8 has been demonstrated to bind specific regions depending on the presence of HS and CS and disruption of the GAG binding domain of CXCL8 or the removal of HS and CS leads to a significant reduction in the detection of this chemokine (Frevert et al., 2003). Moreover, increased concentrations of GAGs have been found in BALF from children with CF (Bhaskar et al., 1998; Hilliard et al., 2007) and secretion of HS (Solic et al., 2005), CS (Khatri et al., 2003; Rahmoune et al., 1991) and hyaluronic acid (Sahu, 1980; Wyatt et al., 2002) is markedly increased in bronchial cells and CF tissues. GAGs have also been shown to

influence the chemokine profile of the CF lung by binding and stabilizing CXCL8 in its active form thus promoting the activation and infiltration of neutrophils (Reeves et al., 2011b; Schlorke et al., 2012).

PA401 is a recombinant therapeutic protein developed by ProtAffin Biotechnologie AG using its patented CellJammer® technology, an engineering platform used to introduce heightened GAG binding affinity into wild type GAG binding proteins and to disrupt receptor binding function (Adage T, 2012; Adage et al., 2012). CXCL8 was chosen as a target for this engineering strategy due to its association with HS and its involvement in numerous neutrophil driven inflammatory diseases including CF, chronic obstructive pulmonary disorder (COPD), rheumatoid arthritis, psoriasis and Crohn's disease (Yang et al., 1999). The recombinant CXCL8 decoy molecule is engineered with a deletion of the first six amino acids including the ELR motif which is essential for receptor binding of human CXCL8. Therefore, disruption of the protein sequence in this area inhibits the chemokine's interaction with its specific G protein-coupled receptors CXCR1 and CXCR2 on leukocytes. In addition, non-crucial amino acids in the GAG binding site were replaced with basic amino acid residues to increase the GAG binding affinity of the chemokine (Adage et al., 2012). With these engineered mutations the novel CXCL8 decoy is capable of rapid binding to endothelial GAGs without binding to GPCRs, thus displacing wild type CXCL8 (Bedke et al., 2010). The aim of the present study was to investigate the ability of PA401 to disrupt the interaction between native CXCL8 and protective GAGs in BALF samples of people with CF, thus rendering the chemokine susceptible to clearance. Some of the results of this study have been previously reported in abstract form (McElvaney OJ, 2013).

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents were purchased from Sigma Aldrich Chemical Co. Ltd., Dublin, Ireland unless indicated otherwise. PA401 (CXCL8 Δ6 F17K F21K E70K N71K) was generated by ProtAffin Biotechnologie AG, Graz, Austria, according to published protocols (Falsone et al., 2013).

2.2. Patient recruitment and sample collection

Patients with CF homozygous for the *ΔF508* mutation (n=13; age 23.6 ± 4.13 mean ± SD; % forced expiratory volume in 1 second (FEV1) 56.3 ± 21.65 predicted) were recruited to this study. BALF samples were collected from patients attending Beaumont Hospital and full informed consent was obtained pre-procedure according to a protocol approved by Beaumont Hospital Ethics Committee. BAL was performed by instilling and aspirating 1 ml/kg of sterile normal saline into the lingula and the right middle lobe. Obtained BALF was then centrifuged at 500 x g for 10 min at 4 °C and the cell free supernatant was aliquoted and stored at -80 °C.

2.3. Native PAGE and Western blot analyses.

BALF (20 µg protein) or 2 ng carrier free recombinant human CXCL8 (RhCXCL8; Biovision Inc. Milpitas, CA, USA) as a positive control were subjected to native gel electrophoresis in NativePAGE Novex 4-16 % (w/v) Bis-Tris gels (Innovagen, Lund, Sweden). Gels were run at 130 V for 1.5 h and were stained with Alcian Blue Solution (Merck Millipore) or Azure A chloride stain overnight (Powell et al., 2010) and then destained with ultra pure water.

For Western blot analysis, proteins were transferred onto polyvinylidene difluoride (PVDF) membrane at 30 V for 1.5 h using a wet blotter. RhCXCL8 was loaded in order to visualize migration of the chemokine. Membranes were blocked for 1 h in 3 % (w/v) dry milk

and 1 % (w/v) bovine serum albumin (BSA) in phosphate buffered saline (PBS) containing 0.05 % (v/v) Tween-20 and then incubated overnight in 1 µg/ml monoclonal mouse anti-CXCL8 specific Ab (MAB208; R&D Systems, Abingdon, UK) or 0.3 µg/ml polyclonal rabbit hCAP-18 specific Ab (Innovagen, Lund, Sweden). The secondary Ab were HRP-linked anti-mouse IgG or HRP-linked anti-rabbit IgG (Cell Signalling Technology, Danvers, MA, USA) incubated at room temperature for 1 h. Immuno-reactive protein bands were visualized employing Immobilon Western chemiluminescent horseradish peroxidase (HRP) substrate (Millipore, MA, USA) on the Syngene G:Box chemi XL gel documentation system (Synoptics, Cambridge, UK). Alternatively, immuno-reactive protein bands were visualized employing SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) after exposure to Kodak® X Omat LS Film.

2.4. Surface Plasmon Resonance (SPR) Affinity Measurements

Kd values for PA401 and CXCL8 binding to immobilized HS were determined by SPR measurements at 25 °C on a BiacoreX100 system (GE Healthcare, Uppsala, Sweden) as recently described (Gerlza et al., 2014). In short, PBS plus 0.005 % (w/v) Tween (Merck, Darmstadt, Germany) was used as a running buffer. Biotinylated GAGs were immobilised on C1 sensor chips (GE Healthcare) which were activated with EDC/NHS and coated with neutravidin (0.2 mg/mL in acetate buffer pH 4). For each binding experiment, 7 different concentrations of the respective PA401 or CXCL8 were measured in quadruplicates. Contact times for all injections and dissociations were 120 s at 30 µL/min. The regeneration solution (1 M NaCl) was enclosed directly after each dissociation time with 30 µL/min and 60 s contact time after each cycle. The maximum response signals of protein binding to the GAG surface, corresponding to the plateaus of the respective sensorgrams, were used for Scatchard plot analysis and the calculation of equilibrium Kd values.

2.5. Enzyme linked immunosorbent assay (ELISA) measurements in pooled CF BALF.

The concentration of CXCL8 or PA401 was measured by ELISA conducted in accordance with the manufacturer's instructions (R&D Systems and ProtAffin, respectively). In brief, 96-well plates (Nunc, Naperville, IL) were coated with 100 µl mouse IgG1 anti-human CXCL8/IL-8 (Cat # MAB208, R&D Systems, diluted to 2 µg/ml)) or monoclonal rat anti-PA401 (# 8A12, ProtAffin, diluted to 7.5 µg/ml) in Voller's Solution (15 mM $NaCO_3$, 35 mM $NaHCO_3$, 3 mM NaN_3, pH 9.8)). The plate was covered and incubated at 4 °C overnight. The plate was washed with 200 µl of Washing Buffer (PBS containing 0.1 % (v/v) Tween 20) three times and blocked for 1 h by adding 200 µl of Blocking Buffer (PBS containing 1 % (w/v) BSA) to block non-specific binding sites in the coated wells. Standards and samples (100 µl) were added to each well for 2 h at room temperature. In additional experiments to investigate the effect of GAGs on CXCL8 quantification, 100 pg of CXCL8 was pre-incubated with HS and CS at a ratio of 1:10, respectively, for 20 min prior to addition to the ELISA plate for 2 h. In a subset of experiments protease inhibitors (1 mM phenylmethanesulfonyl fluoride (PMSF), 10 µg/ml Nα-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK) and Complete Mini tablet (Roche, Basel, Switzerland)) were added after this 2 h incubation. The plate was washed with Washing Buffer three times and then incubated with 100 µl HRP-conjugated antibody (human CXCL8/IL-8 biotinylated Affinity Purified PAb was used for both CXCL8 and PA401 ELISAs, R&D Systems, Cat # BAF208) diluted to 200 ng/ml in Blocking Buffer. The plate was incubated at room temperature for 1 h and then washed with Washing Buffer three times. For development, streptavidin-horseradish peroxidase (HRP) conjugate (R&D Systems), followed by 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was employed with absorbance of each well recorded at 405 nm. IL-6 in BALF was determined by sandwich ELISA (R&D Systems) according to the manufacturer's instructions.

2.6. Treatment of BALF samples

To assess the effect of PA401 on CXCL8 BALF levels, CF BALF was pooled (n=8) and diluted using notmal saline (0.9 % w/v NaCl) to achieve a final CXCL8 concentration of 1000 pg/ml. Increasing concentrations of PA401 (0, 50, 100, 200, 400, 600, 800 or 1000 pg (ProtAffin Biotechnologie AG)) were then added in 1 ml reactions. The *in vitro* effect of PA401 was also evaluated by incubating individual CF BALF samples with pre-determined CXCL8 levels with an equal concentration of PA401. Reactions were incubated for 0, 2, 4, 12 and 24 h at 37 °C and at specific time points reactions were frozen. In a subset of experiments prior to freezing, protease inhibitors (1 mM PMSF, 10 µg/ml TLCK and Complete Mini tablet) were added to each sample to halt CXCL8 protein degradation.

In additional experiment, BALF samples containing protease inhibitors (10 µg/ml TLCK, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, 1 mM PMSF) were pooled (n=4) and diluted to contain 1000 pg/ml CXCL8 as measured by ELISA. Samples were either left untreated or treated with 600 pg/ml PA401, 600 pg/ml PA401 with 250 µg/ml HS or 150 µg/ml heparitinase II and 100 µg/ml chondroitinase ABC for 2 h at room temperature. Samples were subjected to native gel electrophoresis and Western blot analysis.

For experiments investigating displacement and degradation of CXCL8 by PA401 pooled BALF samples (n=4) were diluted to contain 1000 pg/ml CXCL8 as measured by ELISA and were treated with 600 pg/ml PA401 for 1, 5, 15, 30, 60 or 120 min at room temperature with and without protease inhibitors. Samples were subjected to native gel electrophoresis to examine displacement of CXCL8 from GAGs or were subjected to SDS-PAGE to determine degradation of CXCL8, followed by Western blot analysis.

2.7. Proteolytic degradation of PA401

Pooled undiluted CF BALF (20 µl) was pre-incubated for 1 h on ice in the presence or absence of specific protease inhibitors (2 µl of: 500 mM ethylenediaminetetraacetic acid (EDTA), 200 mM pefabloc, 500 mM PMSF, 10 µg/ml leupeptin, or 5 mg/ml E64). Subsequently these samples were incubated with PA401 (100 ng) at 37 °C for 2 h. After the reaction time SDS-PAGE loading buffer (5 µl) was added and samples were boiled at 95 °C for 5 min. Denatured samples were subjected to SDS-PAGE in pre-cast NuPAGE 4-12 % (w/v) gradient gels (Invitrogen) and Western blot analysis was performed employing 7.5 µg/ml monoclonal rat anti-PA401 Ab (MAB8A12; ProtAffin). The secondary Ab was HRP-linked anti-rat IgG (Cell Signalling Technology) To examine proteolysis of CXCL8 and PA401 by serine protease, 25 nM (4 ng/20 µl) of either CXCL8 or PA401 were incubated with varying concentrations of proteinase 3 (PR3) or cathepsin G (CathG) ranging from 100 to 800 nM for 2 h at 37 °C. Proteolysis was stopped by adding denaturing SDS-PAGE sample buffer and samples were analysed by Western blot analysis.

2.8. Preparation of human neutrophils and migration assays

Blood was isolated from healthy control volunteers with no respiratory symptoms and who were not receiving medication. Blood was obtained in 7.5 ml heparinised S-monovette tubes (Sarstedt Ltd, Ireland) and neutrophils were purified by dextran sedimentation and Lymphoprep (Axis-Shield PoC AS, Norway) centrifugation (Reeves et al., 2013). Purified cells (2.5×10^5/200 µl) were resuspended in PBS (pH 7.4) containing 5 mM glucose (PBS-G). Purity of isolated neutrophils was validated by flow cytometric analysis using a monoclonal antibody against CD16b, a specific neutrophil marker (Bergin et al., 2014; Saeki et al., 2009). Neutrophil viability was assessed by Trypan Blue exclusion assay and the potential of PA401 to induce neutrophil apoptosis was excluded by using an Annexin V-FITC Apoptosis Kit (BioVision Inc. Milpitas, CA, USA). Results confirmed viability of neutrophils above 98 % and the purity of isolated neutrophils was greater than 96 %.

Migration assays were performed by measuring the percentage of neutrophils migrating towards CF BALF (containing 1000 pg of CXCL8/ml) treated with 0, 200, 400 or 600 pg/ml PA401 for 4 h, by employing a multiwell chemotaxis chamber (Neuro Probe, Inc., USA) and polyvinylprrolidone-free polycarbonate filters (10 μm thick with 5 μm pores). The stimulant (CF BALF ± PA401) was placed in the lower chamber (total volume of 90 μl) and purified neutrophils in the upper chamber (2.5 x 10^5cells/200 μl). Neutrophil chemotaxis was quantified over 30 min at 37 ºC in a humidified atmosphere (5 % CO_2). The chemotaxis chamber was then disassembled and the polycarbonate filter fixed with methanol and cells stained using Speedy Diff solutions (Clin-tech Ltd, Guildford, UK). The number of migrating neutrophils was determined microscopically employing a Nikon Eclipse TS100 microscope with 10 standardized x 400-high power fields counted for each well. For comparative analysis the number of neutrophils migrating in PBS was set at a migration index of 1.

3. Statistical analysis.

Each individual experiment was performed at least in triplicate and repeated on three or more independent occasions. Results are presented as mean ± standard error of the mean (SEM). GraphPad Prism software version 4.03 for Windows was used for statistical analysis of the data (GraphPad Software, San Diego, CA, USA). One Way-ANOVA was performed when comparing three or more groups. Unpaired Student's t-test analysis was used for comparison between two data sets. Statistically significant differences were determined when a P-value was < 0.05, denoted as * or ** for $P<0.01$.

4. Results

4.1. PA401 reduces CXCL8 levels in CF BALF

Increased expression of GAGs, in particular heparan sulphate (HS), has been reported in individuals with CF (Kuschert et al., 1999). This altered expression profile has been linked to the sustained inflammatory response in the CF airways. As chemokine-GAG complexes are stable in gel shift/mobility experiments (Witt and Lander, 1994), in initial experiments we investigated the CXCL8 protein profiles in BALF of individuals with CF using native-PAGE and Western blot analysis. Carrier free RhCXCL8 (8 kDa) illustrated a fast rate of migration by native gel electrophoresis (Fig. 1A), but in contrast CXCL8 present in CF BALF (n=13) ran as a high molecular weight smear, most likely representing a GAG complexed form of the chemokine as previously described (Reeves et al., 2011b). Alcian blue, the most widely used cationic dye for demonstration of GAGs, and Azure A for sulphated material (Powell et al., 2010), confirmed the presence of GAGs in all CF BALF samples tested (Fig. 1B).

We next compared the HS binding affinity of PA401 to CXCL8. The results show clearly that PA401 binds with a significantly (30-fold) higher affinity to HS compared to the wild type chemokine (Fig. 2A). Regarding the binding kinetics of this interaction, we observed that the higher binding affinity of PA401 is the result of a much slower off rate rather than of a faster on rate, the latter of which was very similar between the two proteins (data not shown).

Ensuing experiments investigated the effect of a single concentration of PA401 on the level of CXCL8 in CF-BALF. Pooled CF-BALF diluted to contain a fixed amount of endogenous CXCL8 (1000 pg/ml) was incubated with PA401 (600 pg/ml) at 37 ^{o}C for 2 h and the level of CXCL8 determined by native gel electrophoresis and immune-blotting. As demonstrated in Fig. 2B, reduced CXCL8 immunoband intensity was observed in CF BALF treated with PA401, an effect reversed by pre-exposing PA401 to 100 fold excess HS, thus

verifying the interaction of PA401 with GAG matrices in CF samples. Moreover, as GAGs including HS interact with chemokines such as CXCL8, we evaluated the effect of digesting GAGs on CXCL8 expression. Post treatment of CF-BALF with chondroitinase ABC and heparitinase II, samples were subjected to native gel electrophoresis and Western blot analysis. As illustrated in Fig. 2B, an immunoreactive band corresponding to complexed CXCL8 was absent in samples treated with GAG lyases. Moreover, samples treated with GAG lyases in the presence of protease inhibitors (0.2 M pefabloc SC and Complete Mini tablet) showed greater levels of immuno-reactivity for the free form of CXCL8 (Fig. 2C). To further explore the effect of PA401 on CXCL8 levels in CF BALF a time course was performed by incubating CF BALF, with a predetermined concentration of endogenous CXCL8 (1000 pg/ml), with PA401 (600 pg/ml) for 1, 5, 15, 30, 60 or 120 min at 37 ^{o}C. By use of a monoclonal antibody for the detection of CXCL8, results of Western blot analysis demonstrated that BALF levels of CXCL8 decreased over time as visualized on both native (Fig. 2D; top panel) and denaturing gels (Fig. 2D; bottom panel). Collectively, these results demonstrate the interaction of CXCL8 with GAGs in CF airway samples, and the reduction in the level of CXCL8 detected post exposure to PA401 forms the foundation of this study.

As immunoblots offer only semiquantitative data, we next investigated the effect of PA401 on the levels of CXCL8 in CF-BALF by ELISA. Pooled CF-BALF diluted to contain a fixed amount of native CXCL8 (1000 pg/ml) was incubated with increasing concentrations of PA401 ranging from 50 pg/ml to 1000 pg/ml at 37 ^{o}C for 0, 2, 4 or 12 h and the level of CXCL8 in each reaction determined by ELISA. Results illustrated that CF BALF treated with PA401 had significantly less CXCL8 than untreated samples ($P<0.01$ at the 2 h time point, n=3), with negligible levels of CXCL8 detected after 12 h incubation in all samples (Fig. 3). A concern in relation to this experiment was the natural degradation of CXCL8 observed in untreated samples thereby skewing data towards possibly overestimating the effect of PA401.

At the 0 h time point the concentration of CXCL8 detected in samples containing 1000 pg/ml PA401 had decreased to 12 % of the untreated sample and it was postulated that this effect may in part be due to continued degradation of CXCL8 during the 2 h antigen-capture phase of the ELISA.

To challenge this hypothesis, CF BALF containing 1000 pg/ml CXCL8 was incubated with an equal concentration of PA401 and after 2 h incubation protease inhibitors were added prior to ELISA for CXCL8 quantification (Fig. 4A). In agreement with previous data, addition of PA401 (1000 pg/ml) significantly reduced the level of CXCL8 to 7.2 % of the untreated control sample ($n=3$; $P<0.01$). In comparison however, inclusion of protease inhibitors (0.2 M pefabloc and complete mini tablets) lessened the observed effect of PA401, although this was still significantly reduced compared to untreated control samples (52 % reduction; $P<0.01$). Ensuing experimental results demonstrated that addition of protease inhibitors immediately after the incubation period at 37 ^{o}C prevented the additional proteolysis of CXCL8 during the ELISA incubation (Fig. 4B). A dose dependent decrease in BALF CXCL8 levels was evident following a 2 h incubation with PA401, that reached a plateau at 600 pg/ml (Fig. 4B). The concentration of CXCL8 at the 2 h time point was significantly decreased by 42 % following exposure to 600 pg/ml PA401 compared to the cytokine level detected in untreated BALF ($P<0.05$). Of importance, a comparison of the quantification of CXCL8 (100 pg) pre-exposed to a mixture of both HS and CS was performed (Fig. 4C). ELISA results revealed that detection of CXCL8 was significantly decreased when incubated with GAGs at a ratio of 1:10 ($P<0.05$). These results are in line with previously published data (Reeves et al., 2011b) and demonstrate that levels of CXCL8 in CF BALF samples, and the effectiveness of PA401, may be underestimated.

In the described experiments we have confirmed the effect of PA401 in pooled CF BALF and therefore in subsequent tests individual, undiluted, BALF samples were employed

(Fig. 5). PA401 was added to CF BALF at the equivalent predetermined CXCL8 concentration. Results indicate that PA401 significantly reduced CXCL8 levels in all CF BALF samples tested but the effect varied between individual patients (n=4). At 4 h post PA401 treatment the concentration of CXCL8 in CF#1 BALF decreased by 35 % (P=0.01), CF#2 BALF decreased by 29 % (P=0.05), CF#4 decreased by 15 % (P=0.05) and CF#5 BALF levels of CXCL8 decreased by 33 % (P=0.01) (Fig. 5). In control experiments the PA401 decoy used in assays (0-1000 pg/ml) was not detected by the CXCL8 ELISA confirming no cross reactivity between the decoy and native CXCL8 in pooled CF BALF. PA401 was found to have no effect on BALF levels of other inflammatory mediators including IL-6 or the anti-microbial peptide hCAP-18 (result not shown).

4.2. PA401 is proteolytically degraded in CF BALF.

Quantification of PA401 by ELISA employing a monoclonal rat anti-PA401 (# 8A12, ProtAffin) revealed that the concentration of decoy present in CF BALF decreased as the time of incubation increased (Fig. 6A). Specifically, at the 2 h time point the level of PA401 present in samples initially containing 1000 pg/ml had decreased by 53 % ($P<0.05$). These results indicate that the decoy can quickly lead to the degradation of native CXCL8 in CF BALF, and in turn, is subsequently degraded and thus build-up of the recombinant protein is unlikely. Western blot analysis was used to elucidate the protease(s) responsible for degradation of PA401 in CF BALF. PA401 (100 ng) was incubated for 2 h at 37 °C in pooled undiluted CF BALF followed by separation by SDS-PAGE and Western blot analysis employing monoclonal rat anti-PA401 Ab. An immuno-reactive band for PA401 was clearly visible at approximately 8 kDa. In contrast however, degradation of the decoy was evident after 2 h incubation in CF BALF (Fig. 6B). To identify protease(s) responsible for cleavage and destruction of PA401 in CF BALF, various protease inhibitors were pre-incubated with

CF BALF before adding PA401. The use of broad range protease inhibitors targeting elastase-like, trypsin-like and cysteine-like proteases and metalloproteases identified elastase-like proteases as playing a key role in the proteolytic cleavage of PA401. As shown in Fig. 6B, PMSF and pefabloc inhibited the cleavage of PA401, whereas leupeptin and E64 (trypsin- and cysteine-like), and EDTA (metalloproteinase-like) had no or little effect. Taken together, these results show that elastase-like proteases may be involved in the degradation of PA401 within the CF lung. It has previously been shown that neutrophil elastase is capable of degrading CXCL8 (Leavell et al., 1997) and for this reason we examined the ability of other neutrophil derived serine proteases to cleave CXCL8 and PA401. A dose response experiment was performed by incubating RhCXCL8 or PA401 (25nM) with PR3 or CathG (100, 200, 400 or 800 nM) for 2 h at 37 ^{o}C. Fig. 6C shows that levels of RhCXCL8 and PA401 detected by Western blot analysis decreased with increasing enzyme concentrations and 400 nM fully degraded the chemokine and decoy after 2 h incubation. No fragmentation products were detected for either PA401 or RhCXCL8 by immunoblotting. These results confirm that CXCL8 and PA401 are equally susceptible to proteases present within the CF airways.

4.3. CF BALF induced neutrophil chemotaxis is reduced in cells exposed to PA401 treated BALF.

Neutrophilic inflammation is partially explained by the stability of CXCL8 within the CF lung and binding to HS enhances neutrophil responses to CXCL8 (Webb et al., 1993). For this reason we investigated the impact of PA401 induced clearance of CXCL8 on associated neutrophil migration *in vitro*. Firstly, the robustness of this assay was demonstrated by measuring the dose-dependent migration of neutrophils towards CXCL8 (1 or 10 ng) (Fig. 7A). Subsequently, pooled CF BALF (n=4) containing 1000 pg/ml CXCL8 was exposed to

PA401 (0, 200, 400 or 600 pg/ml) for 4 h. The migration index of healthy control neutrophils in response to CF-BALF was compared pre- and post-PA401 treatment with results illustrating a significant 16% ($P<0.05$) decrease in migration activity in samples exposed to 400 pg/ml of PA401 compared to untreated BALF. At a higher concentration, PA401 (600 pg/ml) reduced the neutrophil migration index produced by CF BALF by 25 % ($P<0.05$) (Fig. 7B).

Taken together, these *in vitro* results indicate that GAG-immobilised CXCL8 maintains activity and *in vitro*, post-PA401 treatment, CXCL8 is solubilised from GAGs rendering it susceptible to proteolysis. PA401 itself is also susceptible to degradation which is of little consequence as the beneficial effects of decreased levels CXCL8 are already evident by the weakened chemotactic capacity of CF-BALF.

5. Discussion

The aim of this study was to evaluate the effect of a novel CXCL8 decoy molecule on native CXCL8 levels in CF BALF. Inflammation in the CF lung is mediated in part by the presence of increased concentrations of GAGs which stabilise active CXCL8 and protect it from proteolytic degradation, therefore establishing a chemotactic gradient and promoting the influx of destructive neutrophils (Kuschert et al., 1999). The GAG most successful at binding CXCL8 is heparan sulphate when in association with the core protein syndecan-1 (Marshall et al., 2003). Potential therapeutics that directly target inflammatory processes and remove the need for prolonged use of corticosteroids are highly desirable (Konstan and Davis, 2002). Inflammation that accompanies mucus accumulation in patients with CF is currently treated using a variety of aerosolized therapies (Konstan and Davis, 2002). Nebulized HTS contributes to rehydration of the airways and increases mucociliary clearance, and additionally reduces chemokine levels in CF BALF via the disruption of ionic interactions with GAGs (Reeves et al., 2012; Reeves et al., 2011b). Results of the present study indicate that clinical application of a CXCL8 decoy may serve to decrease the inflammatory burden in the CF lung *in vivo*.

The effects of PA401 have been previously analysed using an *in vitro* flow system of leukocyte recruitment to primary human microvascular endothelium (Bedke et al., 2010). Application of the CXCL8 decoy molecule was found to inhibit neutrophil rolling and adherence. A similar experimental set up was used to analyse the effect of PA401 on monocyte adhesion employing the HDMED cell line. Under these experimental conditions the decoy caused decreased interaction between endothelial cells and monocytes and shear resistant adhesion of mononuclear cells to the endothelium was reduced (Bedke et al., 2010). The compound was also assessed in an *in vivo* model for kidney ischemia/reperfusion (I/R) and acute renal allograft damage in rats. Infiltration of neutrophils to the site of I/R has been

implicated in the acute rejection of transplanted allograft organs (Hirayama et al., 2006) and treatment with PA401 reduced acute damage to allograft kidneys (Bedke et al., 2010). The positive outcomes of these investigations lead to PA401 being granted orphan medicinal product status for use in delayed graft function after solid organ transplant by the EMA and FDA. The properties of PA401 have also been investigated in the context of inflammatory lung disease using a mouse model (Adage T, 2012). The subcutaneous administration of PA401 reduced the level of neutrophil infiltration detected in the BALF of chronic smoke induced and acute LPS induced models of murine lung inflammation (Adage et al., 2012). A murine model of mBSA (methylated BSA) - induced arthritis has been utilised to evaluate the systemic anti-inflammatory potential of PA401. The decoy was administered subcutaneously resulting in a decrease in neutrophil infiltration to the knee cavity as measured in knee lavage fluid (Falsone et al., 2013). The results obtained from animal studies thus far have been achieved by delivering the decoy intravenously. However, as the proposed mode of delivery for treatment of lung inflammation is via inhalation we sought to discover the effects of the decoy when directly applied to BALF of patients with CF. Within this study we have shown that exposure of pooled CF BALF to PA401 lead to a reduction in the concentration of native CXCL8 detected in CF BALF samples. The proposed mode of action for PA401 within the airways is the ability of the decoy to disrupt CXCL8:GAG interactions thus rendering native CXCL8 susceptible to proteolytic degradation by elastase like proteases. We have previously shown that upon release of CXCL8 from GAG matrices by HTS the chemokine is rendered unprotected and exposed to proteolysis by neutrophil elastase (Reeves et al., 2011b). This observation is also supported by work by Leavell *et al*., (1997) who demonstrated that incubation of CXCL8 with neutrophil elastase resulted in the loss of CXCL8 chemotactic activity in a dose- and time-dependent fashion, an effect incurred by proteolysis of CXCL8 into undetectable small fragments (Leavell et al., 1997).

Despite the potential advantages of delivery of PA401 to the lungs, *in vivo* models would be necessary to evaluate presence of the decoy on the respiratory epithelial surface and penetration into the interstitium following aerosolization. One of the potential challenges with administration of PA401 is that the decoy itself is susceptible to degradation by proteases present in the airways. This occurrence may be prevented by rational design to improve the PA401 molecule rendering it less susceptible to proteolysis, but only if bioactivity is not influenced by this additional engineering. Indeed, the susceptibility of the drug to degradation closely mirrors challenges associated with aerosolization of recombinant secretory leukocyte protease inhibitor (SLPI) to the lungs of individuals with CF. SLPI is a potent inhibitor of neutrophil elastase, however, it has been shown that this antiprotease is vulnerable to degradation and inactivation by cysteinyl cathepsins (Taggart et al., 2001) and serine proteases, (Weldon et al., 2009) and thus the utilization of SLPI as effective therapy for airways disease presents some challenges. A solution to this conundrum was delivery of recombinant SLPI via a liposomal carrier which successfully protected the activity of SLPI against cathepsin L mediated proteolysis, whilst having no detrimental effect on SLPI access to intracellular sites of action *in vitro* (Gibbons et al., 2009). Such an approach may also prove useful in aerosolization of PA401 to prolong its activity and further studies would be required for confirmation.

The results of this study demonstrate that as a consequence of PA401 exposure and reduced levels of CXCL8, there is a decrease in neutrophil chemotaxis in response to CF BALF. CXCL8 is a major chemotactic factor in CF and lowering the levels of CXCL8 in the CF airways could be expected to somewhat decrease neutrophil chemotaxis. It is however only one of a number of chemoattractants found in the CF airways. Leukotriene B_4, formyl peptides, C5a, platelet activating factor (Mackerness et al., 2008) and more recently proline-glycine-proline (Gaggar et al., 2008) are among other chemoattractants that contribute to

neutrophil influx. Nevertheless, in the present study we have demonstrated that disruption of GAG:CXCL8 interactions in CF BALF samples by PA401 was sufficient to significantly decrease the number of migrating neutrophils *in vitro*, with the potential for an effective anti-inflammatory outcome *in vivo*.

6. Conclusion

In summary, treatment to specifically target chronic inflammation exacerbations in CF has largely been achieved with the use of inhaled medications such as antibiotics, corticosteroids and mucolytics (Flume et al., 2009). The CXCL8 decoy PA401 disrupts the interaction between GAGs and CXCL8, rendering CXCL8 susceptible to proteolytic degradation with subsequent decrease in neutrophil chemotaxis. Consequently, this decoy approach represents a novel way to treat the high levels of GAG bound CXCL8 present in the CF lung, which is a major contributing factor to lung morbidity in patients.

Authorship contributions

Contribution: E.P.R., N.G.M., T.A. and A.J.K., conceived and planned the study design and designed experiments; O.J.M., N.O.R., M.W., N.L., K.P., E.P.R. and N.G.M. performed quality assurance, interpreted the data and wrote the manuscript; O.J.M., N.O.R., M.W., N.L., K.P., T.G., D.A.B. and H.K. carried out experiments and performed statistical analysis. C.M. and M.E.O.B. contributed to patient accrual and performed clinical data collection.

Conflict of interest disclosure: The authors declare no competing financial interests.

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Figure Legends

Fig. 1. GAG expression profile and pattern of CXCL8 in BALF of individuals with CF.

Native gel electrophoresis and Western blot analysis for CXCL8 in CF BALF (n=7). Carrier free recombinant human CXCL8 (Rh-CXCL8) was employed as a control to visualize protein migration. Western blot analysis confirmed that CXCL8 exists in a high molecular weight complex in CF BALF when compared to Rh-CXCL8. B; Native gels of CF BALF stained with Alcian Blue (top panel) or Azure A chloride stain (bottom panel). The Western blot and stained gels illustrated are representative results of one out of three separate experiments.

Fig. 2. Levels of CXCL8 decrease in CF BALF following exposure to PA401.

A; Kd values for PA401 and CXCL8 binding to immobilized HS determined by SPR. PA401 binds with a significantly (30-fold) higher affinity to HS compared to the wild type chemokine. B; Pooled BALF samples containing 1000 pg/ml endogenous CXCL8 was either left untreated or was treated with PA401 (600 pg/ml), PA401 preexposed to HS (250 µg/ml) or GAG lyases (heparanase (150 µg/ml) and chondroitinase (100 µg/ml)) for 2 h. Samples were subjected to native gel electrophoresis and Western blot analysis for CXCL8. C; CF BALF with and without GAG lyases and with protease inhibitors (0.2 M pefabloc SC and Complete Mini tablet) was subjected to native gel electrophoresis and Western blot analysis for CXCL8. The position of free and complexed forms of CXCL8 is indicated. D; Pooled CF BALF (n=4) was exposed to PA401 for 1, 5, 15, 30, 60 or 120 min and subjected to native (top panel) or denaturing (bottom panel) gel electrophoresis and Western blot analysis for CXCL8. The Western blots illustrated are representative results of one out of three separate experiments.

Fig. 3. Decreased CF BALF levels of CXCL8 post exposure to PA401 confirmed by ELISA.

Pooled CF BALF (n=4) was exposed to increasing concentrations of PA401 for 0, 2, 4 or 12 h and levels of CXCL8 were determined by ELISA. Statistical analysis across individual time points performed by One Way-ANOVA (*=P<0.05; **=P<0.01).

Fig. 4: Detection of reduced levels of CXCL8 in CF BALF supplemented with proteases inhibitors post PA401 exposure.

A; CF BALF (containing 1000 pg of CXCL8/ml) was either untreated or treated with protease inhibitors (PI; 0.2 M pefabloc and Complete Mini tablets) and subsequently either untreated or treated with PA401 (1000 pg) for 4 h. CXCL8 levels were detected by ELISA and expressed as pg/ml. (**P<0.01 by Student's t-test; NS = no significant difference. n=4 independent experiments). B; Pooled CF BALF (n=4) was exposed to PA401 for 0, 1, 2 or 4 h at increasing concentrations (0-600 pg/ml). After the indicated time points PI were added to prevent further CXCL8 degradation and levels were detected by ELISA (*P<0.05, **P<0.01 by ANOVA; n=4 independent experiments). C; ELISA analysis for CXCL8 (100pg) in the absence or presence of GAGs (1:10 ratio). Bars indicate standard error of the mean (**P<0.01 by Student's t-test; n=3 independent experiments).

Fig. 5. PA401 decreased CXCL8 levels in individual CF BALF samples.

PA401 was added at a concentration equivalent to the predetermined level of CXCL8 in each undiluted CF BALF sample (n=4). Reactions were incubated at 37 ^{o}C for 4 h followed by the addition of PI and CXCL8 quantification by ELISA (Student's t-test, **P<0.01, *P<0.05 between treated and untreated respective samples; NS = non-significant). Bars indicate the mean ± SEM of n=3 independent experiments.

Fig. 6. PA401 is proteolytically degraded in CF BALF.

A; PA401 levels in pooled CF BALF (n=4) decreased over time as detected by ELISA employing a rat anti-PA401 Ab (*P<0.05, **P<0.01 by Student's t-test between the 1000 pg/ml at time 0 h and 2 h or 4 h respectively; n=3 independent experiments). B: Immuno-blot showing PA401 (100 ng/ 20 µl) degradation in pooled CF BALF (n=6). PA401 was incubated in PBS as a negative control or CF BALF as a positive control (Con). CF BALF samples (20 µl) were pre-incubated with 2 µl of protease inhibitors; EDTA (500 mM), PMSF (500 mM), pefabloc (Pef, 200 mM), Leupeptin (Leu, 10 mg/ml) or E64 (5 mg/ml). Subsequently, samples were incubated with PA401 (100 ng) at 37 °C for 4 h. PA401 breakdown was primarily prevented by inclusion of elastase like inhibitors. C; Western blot analysis of the proteolytic degradation of RhCXCL8 and PA401 (25nM) by PR3 and CathG (100-800nM) for 2 h at 37 ^{o}C. Results presented in B and C are representative blots of 3 separate experiments.

Fig. 7. PA401 reduced CF BALF induced neutrophil migration.

A; Mean migration index of purified neutrophils in response to CXCL8 (0, 1 or 10 ng). B; Induced mean migration index of purified neutrophils ($2x10^5$ /200 µl) in response to CF BALF (containing 1000 pg of CXCL8/ml) was reduced post treatment with PA401 (200, 400 or 600 pg/ml) for 4 h when compared to the untreated control. For comparative analysis cells in PBS were set as a migration index of 1. Three experiments each with five replicates were performed on separate days. Bars indicate standard error of the mean (*P<0.05 by Student's t-test).

Figure 1

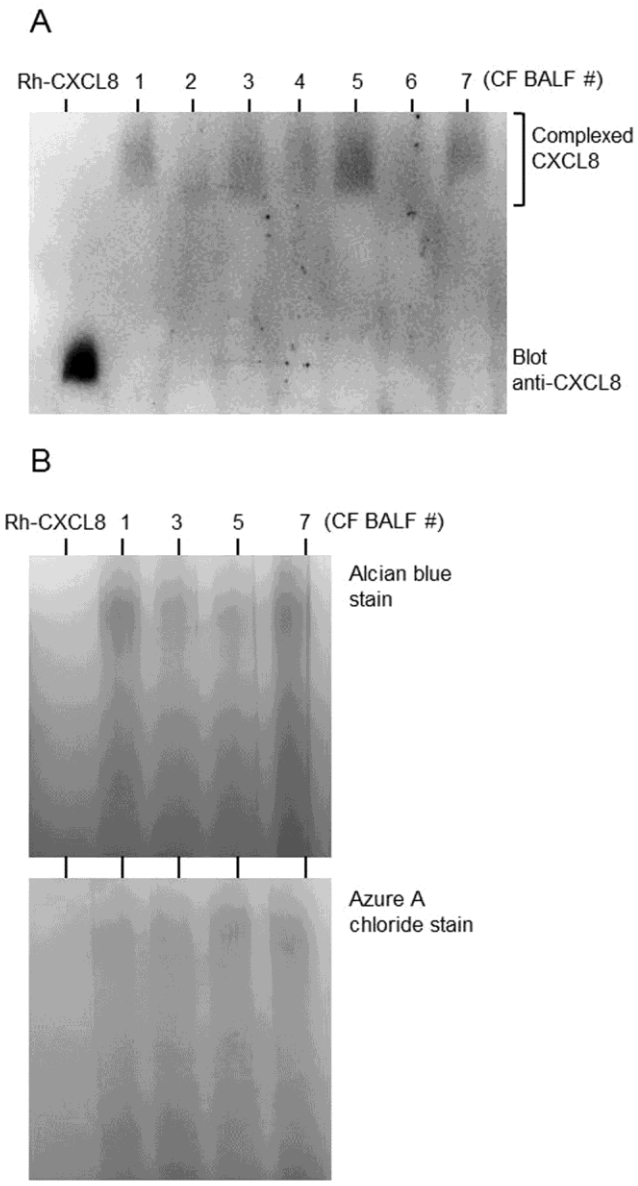

A
Rh-CXCL8 1 2 3 4 5 6 7 (CF BALF #)
Complexed CXCL8
Blot anti-CXCL8
B
Rh-CXCL8 1 3 5 7 (CF BALF #)
Alcian blue stain
Azure A chloride stain

Figure 2

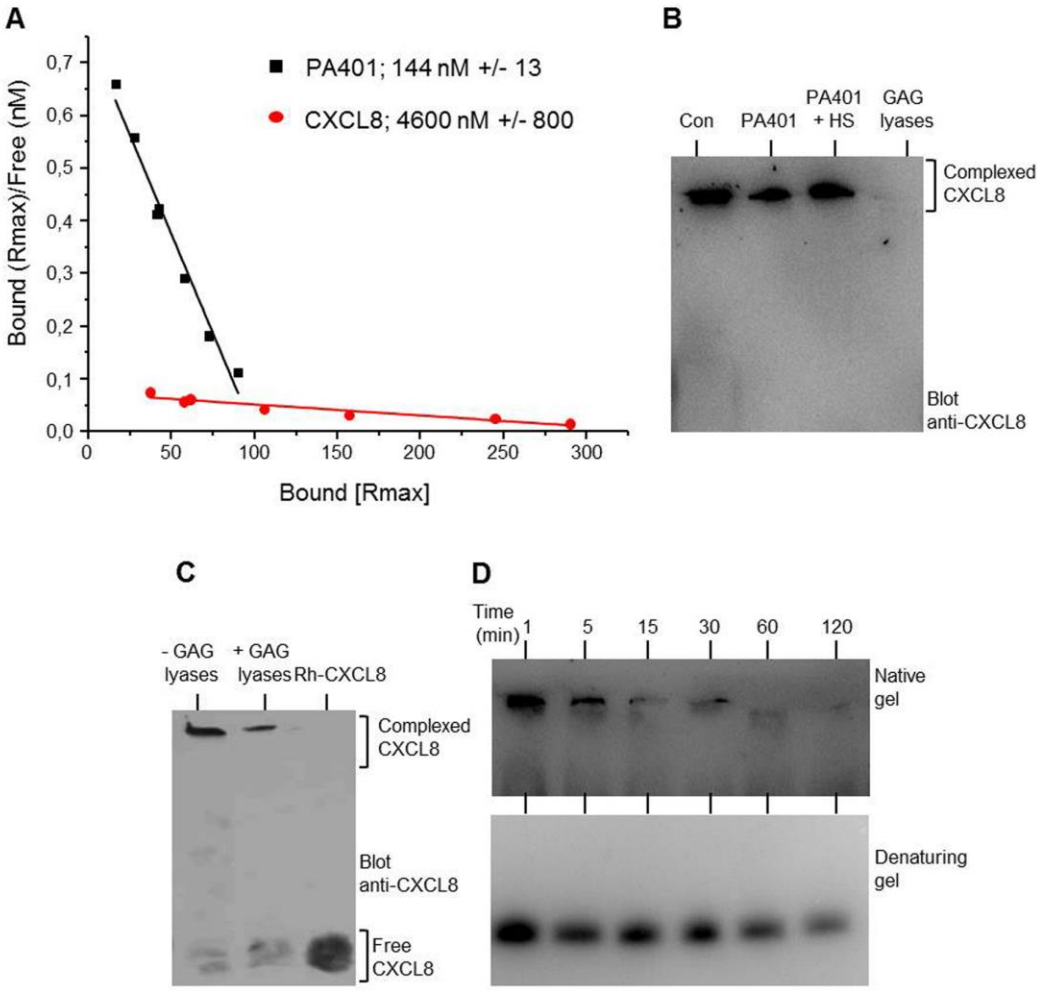

A
PA401; 144 nM +/- 13
CXCL8; 4600 nM +/- 800
Bound (Rmax)/Free (nM)
Bound [Rmax]
B
Con
PA401
PA401 + HS
GAG lyases
Complexed CXCL8
Blot anti-CXCL8
C
- GAG lyases
+ GAG lyases
Rh-CXCL8
Complexed CXCL8
Blot anti-CXCL8
Free CXCL8
D
Time (min)
1
5
15
30
60
120
Native gel
Denaturing gel

Figure 3

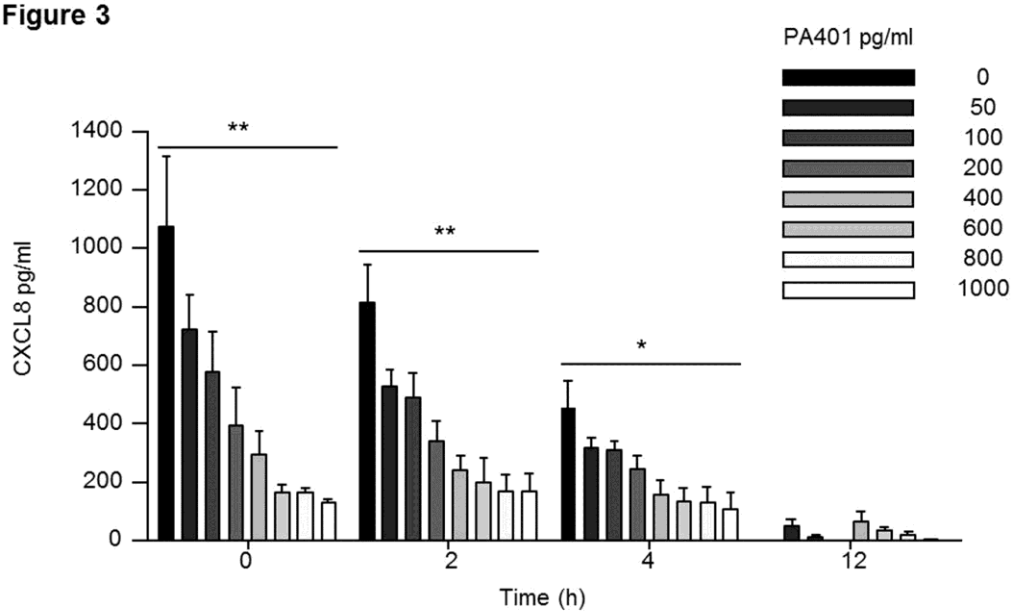

PA401 pg/ml
0
50
100
200
400
600
800
1000
CXCL8 pg/ml
1400
1200
1000
800
600
400
200
0
**
**
*
0
2
4
12
Time (h)

Figure 4

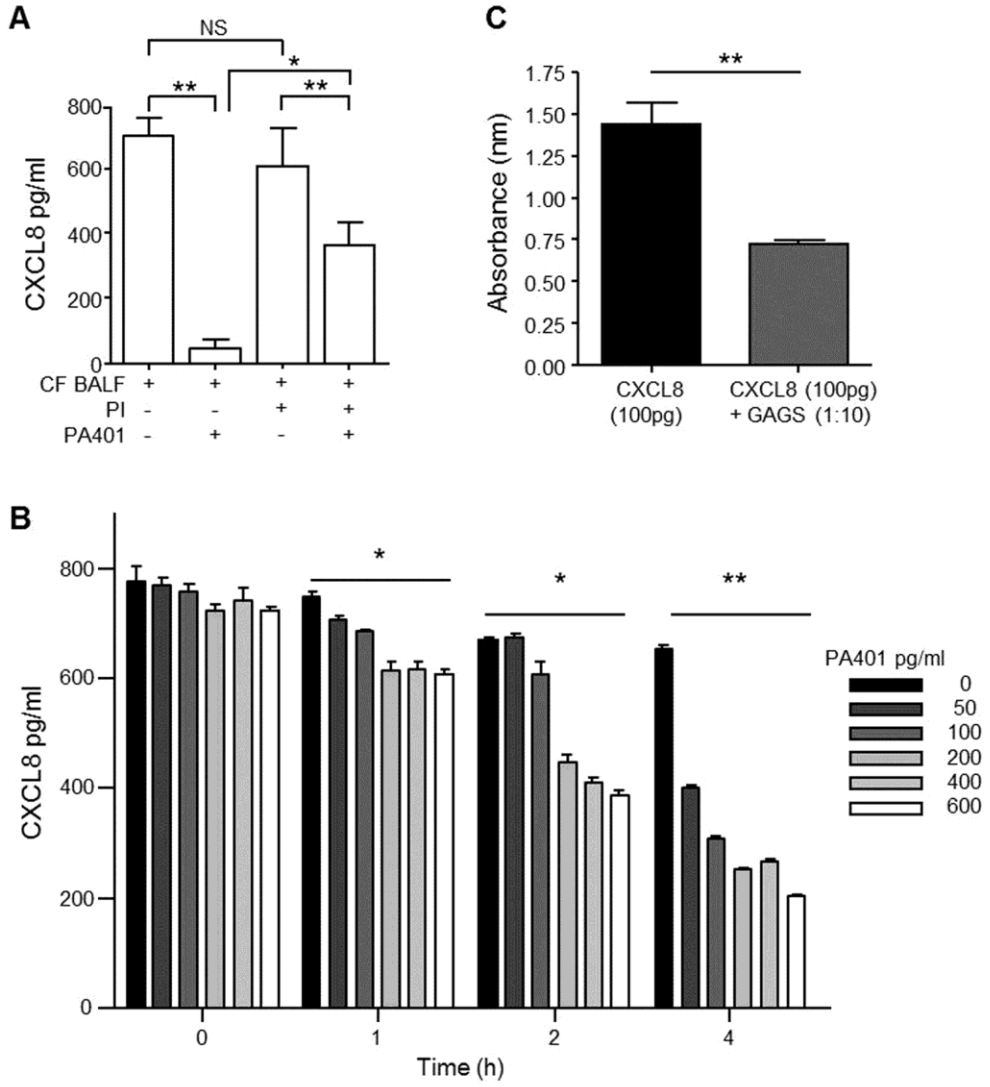

A
NS
*
**
**
800
600
400
200
0
CXCL8 pg/ml
CF BALF + + + +
PI - - + +
PA401 - + - +
C
**
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Absorbance (nm)
CXCL8 (100pg)
CXCL8 (100pg) + GAGS (1:10)
B
*
*
**
800
600
400
200
0
CXCL8 pg/ml
0
1
2
4
Time (h)
PA401 pg/ml
0
50
100
200
400
600

Figure 5

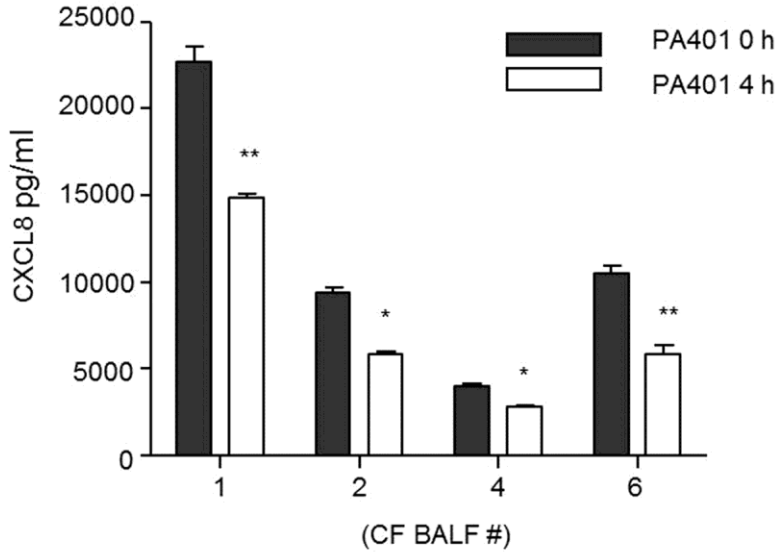

25000
20000
15000
10000
5000
0
CXCL8 pg/ml
PA401 0 h
PA401 4 h
**
*
*
**
1
2
4
6
(CF BALF #)

Figure 6

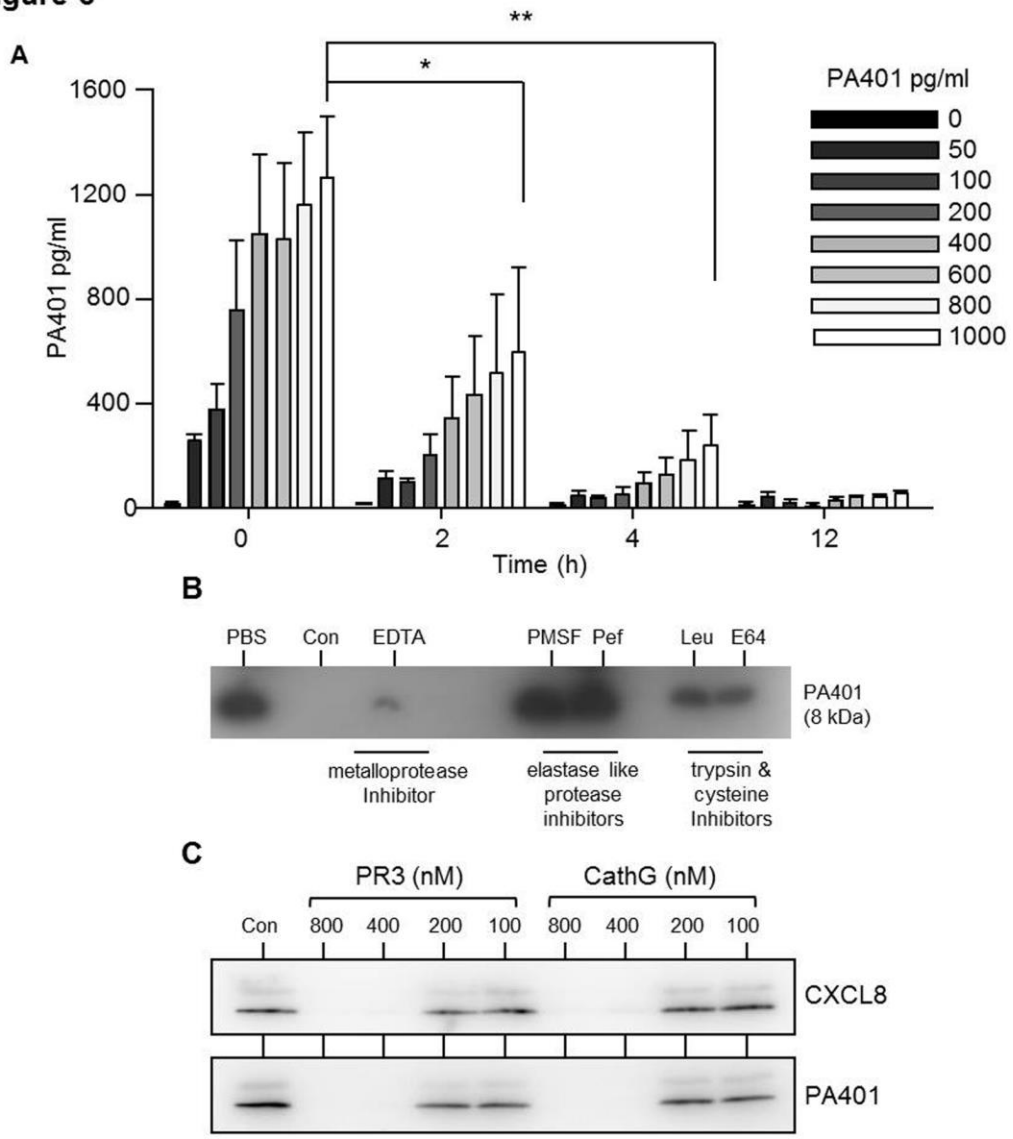
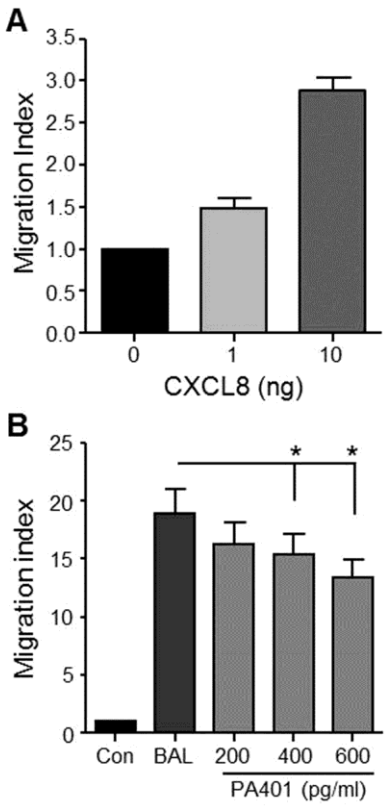

A
**
*
PA401 pg/ml
0
50
100
200
400
600
800
1000
1600
1200
800
400
0
PA401 pg/ml
0
2
4
12
Time (h)
B
PBS
Con
EDTA
PMSF
Pef
Leu
E64
PA401
(8 kDa)
metalloprotease
Inhibitor
elastase like
protease
inhibitors
trypsin &
cysteine
Inhibitors
C
PR3 (nM)
CathG (nM)
Con
800
400
200
100
800
400
200
100
CXCL8
PA401

Figure 7


A
Migration Index
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
1
10
CXCL8 (ng)
B
Migration index
25
20
15
10
5
0
*
*
Con
BAL
200
400
600
PA401 (pg/ml)